Arch. Microbiol. 125, 203–207 (1980)

Archives of
Microbiology

Oxidation of Biphenyl by the Cyanobacterium, *Oscillatoria* sp., Strain JCM

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Abstract. The oxidation of biphenyl by Cyanobacterium, *Oscillatoria* sp., strain JCM was studied. The organism grown photoautotrophically in the presence of biphenyl oxidized biphenyl to form 4-hydroxybiphenyl. The structure of the metabolite was elucidated by ultraviolet and mass spectra and shown to be identical to authentic 4-hydroxybiphenyl. In addition this metabolite had properties identical to 4-hydroxybiphenyl when analyzed by thin-layer and high-pressure liquid chromatography. Experiments with [^{14}C]-biphenyl showed that over a 24 h period the organism oxidized 2.9 % of the added biphenyl to ethyl acetate-soluble products.

Key words: Biphenyl oxidation – Cyanobacterium – *Oscillatoria* – 4-hydroxybiphenyl formation.

Several authors have reported the ubiquitous occurrence of bacteria which can utilize biphenyl as sole source of carbon and energy (Ahmed and Focht, 1973; Catelani et al., 1971; Lunt and Evans, 1970; Ohmori et al., 1973; Gibson et al., 1973). Lunt and Evans (1970) demonstrated that a gram negative soil bacterium oxidized biphenyl to 2,3-dihydroxybiphenyl, α-hydroxy-β-phenyl muconic semialdehyde and phenyl pyruvic acid. Further studies (Catelani et al., 1971, 1973; Catelani and Colombi, 1974) indicated that a strain of *Pseudomonas putida* oxidized biphenyl to benzoic acid via 2,3-dihydro-2,3-dihydroxybiphenyl, 2,3-dihydroxybiphenyl and 2-hydroxy-6-oxo-phenylhexa-2,4-dienoic acid. Gibson et al. (1973) provided further evidence for initial oxidative attack at the 2,3-position on the biphenyl molecule. They isolated a mutant strain, *Beijerinckia* B8/36 that oxidized biphenyl to cis-2,3-dihydroxy-1-phenylcyclohexa-4,6-diene (cis-2,3-dihydro-2,3-dihydroxybiphenyl). The identification of cis-2,3-dihydro-2,3-dihydroxybiphenyl suggests a dioxygenase catalyzed reaction (Gibson, 1977).

In contrast to bacteria, fungi oxidize biphenyl primarily at the 4-position to form 4-hydroxybiphenyl and 4,4′-dihydroxybiphenyl (Smith and Rosazza, 1974; Wiseman et al., 1975; Dodge et al., 1979). These results are similar to those reported for the mammalian metabolism of biphenyl (Raig and Ammon, 1970; Meyer and Scheline, 1976; Wiebkin et al., 1976; Meyer et al., 1976).

Recently we demonstrated that the ability to oxidize naphthalene is widely distributed throughout the algal kingdom (Cerniglia et al., 1979, 1980a, b). In view of the environmental implications of these findings, we tested the capacity of algae to metabolize other aromatic hydrocarbons.

In this study, the oxidation of biphenyl by the cyanobacterium, *Oscillatoria* sp., strain JCM is described.

Materials and Methods

Organism and Culture Conditions

Oscillatoria sp., strain JCM was maintained in the light on slants of ASP-2 medium (Van-Baalen, 1962) containing 8 μg/l of vitamin B_{12}. Medium ASP-2 contains in g/l: NaCl, 18.0; $MgSO_4 \cdot 7H_2O$, 5.0; KCL, 0.60; $CaCl_2 \cdot H_2O$, 0.37; $NaNO_3$, 1.0; KH_2PO_4, 0.05; Tris buffer, 1.0; and 1 ml of a trace metals solution containing in mg/l: H_3BO_3, 3430; $CuSO_4$, 0.3; $CoCl_2 \cdot 6H_2O$, 1.2; $MnCl_2 \cdot 4H_2O$, 432; $ZnCl_2$, 31.5; MoO_3(85 %), 3.0. The initial pH was 8.2. The organism was incubated at 39 ± 0.1 °C in test tubes (22 × 175 mm) that contained 20 ml of ASP-2 medium. Air enriched with 1.0 ± 0.1 % (v/v) CO_2 was continuously bubbled through the tubes. Illumination was provided by two fluorescent lamps (F48T12/CW/Ho, General Electric, Cleveland, Ohio). The lamps were placed on each side of the water bath at a distance of 8.5 cm from the center of the test tubes.

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Abbreviations: tlc – thin-layer chromatography; hplc – high pressure liquid chromatography

0302-8933/80/0125/0203/$01.00

Biotransformation Studies

Biotransformation experiments were conducted in a 'closed flask' growth system as previously described (Cerniglia et al., 1979, 1980a). In a typical experiment, 30 ml of exponentially-growing cells (0.8 mg/dry wt · ml) were transferred directly from the growth tube to a sterile flask. Biphenyl (0.1 mg in 0.1 ml of 95% ethanol) was added to the culture medium and the flask was sealed. Ethanol had no effect on growth. The flask was clamped to the top of a linear rotating bar with a 5 cm throw and shaken at 40 rev/min. The bottom 3–4 cm of the flask was immersed in a 30°C water bath. The culture was equilibrated for 15 min in the dark during which time CO_2 was added via a syringe through the small sidearm. The carbon dioxide level in the flask was maintained between 0.5 and 1.0% throughout the experiment. Illumination was provided by two fluorescent lamps (F48T12/CW/HO) positioned 29 cm below the water bath. The fluorescent lamps were covered with a yellow filter (3.2 mm thick plexiglass sheet, No. 2422, Rohm and Haas, Philadelphia, Pennsylvania). Five flasks were incubated as described above. Control experiments were also conducted by incubating biphenyl in ASP-2 medium in the absence of *Oscillatoria* sp., strain JCM and also in the presence of cells that had previously been killed by heat treatment. Experiments with [U-^{14}C]-biphenyl (27 nmol, 1.0 μCi) were conducted as described above. All culture volumes were 30 ml.

Extraction of Transformation Products

After 24 h incubation, cultures were examined by light microscopy for bacterial contamination. No bacterial contamination was detected. Cells were removed by filtration through glass wool and the filtrate was extracted with three equal volumes of ethyl acetate. The organic extract was dried over anhydrous Na_2SO_4 and the solvent removed in vacuo. Analysis of the residue was conducted by thin-layer and high-pressure liquid chromatography.

Analytical Methods

Thin-layer chromatography (tlc) was performed with Polygram SilG/UV_{254} plastic sheets (Macherey-Nagel and Co., Düren, FRG). The solvent used for chromatography was chloroform : acetone (8 : 2). Biphenyl metabolites were located on chromatograms by viewing under ultraviolet light (254 nm) and also by their characteristic color with Gibbs reagent (2,6-dichloroquinone-4-chloroimide in methanol [2% v/v]). The radiolabelled extracts were separated by tlc as described above. The radioactive residue was dissolved in 0.1 ml of acetone and a 30 μl sample was applied to the base of the tlc plate. Biphenyl, 4-hydroxybiphenyl, 3-hydroxybiphenyl, 2-hydroxybiphenyl, 2,2′-dihydroxybiphenyl and 4,4′-dihydroxybiphenyl were co-chromatographed with the radiocative extract. The standard compounds were visualized under ultraviolet light. Metabolites were also detected by autoradiography using Kodak X-Ray film (Blue-sensitive SB-54). After 3–5 days the exposed film was processed using Kodak D-19 developer and Kodak rapid fixer to show the location of labelled compounds.

High-pressure liquid chromatography (hplc) was also used to separate and quantitate the amount of each biphenyl metabolite. Hplc was performed on a component system consisting of a Waters Associates Model 440 absorbance detector operated at 254 nm or 280 nm. A μBondapak C_{18} column (3.9 mm × 30 cm) was used for the separation of polar metabolites, which was achieved with a programmed acetonitrile : water linear gradient (30–70%, 30 min). The initial flow rate was 1.5 ml/min. To resolve 2-hydroxy, 3-hydroxy and 4-hydroxybiphenyl, a μporasil column (3.9 mm × 30 cm) was used with a hexane/ethyl acetate (85 : 15 v/v) solvent system. The system was run isocratically for 15 min at a flow rate of 1.0 ml/min. The Waters Associates hplc, μBondapak C_{18} and μporasil columns were purchased from Waters Associates, Inc., Milford, Massachusetts, USA. In the studies with [^{14}C]-biphenyl (0.50 ml) fractions were collected and the radioactivity present in each fraction determined with a Beckman LS-250 liquid scintillation counter. Aquasol-2 (New England Nuclear Corp., Boston, Massachusetts, USA) served as the scintillation fluid. Absorption spectra were determined on a Beckman model 25 recording spectrophotometer (Beckman Instruments, Inc., Palo Alto, California, USA). Low resolution mass-spectra were determined on a DuPont model 21-491 mass spectrometer.

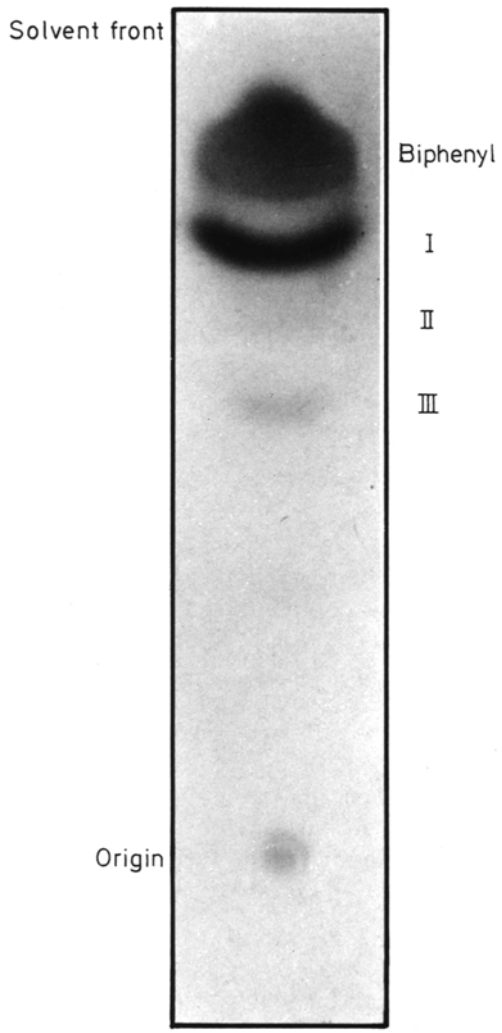

Fig. 1. Autoradiogram of tlc plate showing the metabolites formed from [^{14}C]-*biphenyl* by *Oscillatoria* sp., strain JCM. Procedures are described in the text. Areas on the thin-layer chromatogram that contained radioactivity were arbitrarily designated Compounds I–III as shown

Chemicals

Biphenyl (99.95%) was from Mallinckrodt Chemical Works, St. Louis, Missouri, USA. [U-^{14}C]-biphenyl (specific activity 37 mCi/mmol) was from Amersham/Searle, Arlington Heights, Illinois, USA. 2-Hydroxy, 4-hydroxy, 2,2′-hydroxy and 4,4′-dihydroxybiphenyl were from Aldrich Chemical Company, Milwaukee, Wisconsin, USA. 3-Hydroxybiphenyl was obtained from RFR Corporation, Hope, Rhode Island, USA.

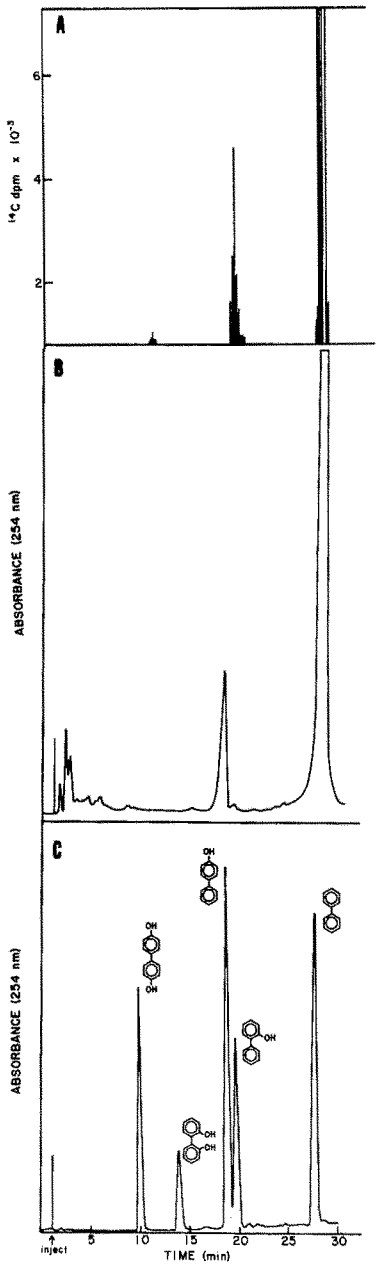

Fig. 2A—C. Hplc analysis of metabolites formed from biphenyl by *Oscillatoria* sp., strain JCM. **A** Radioactive metabolites formed from [^{14}C]-biphenyl by *Oscillatoria* sp., strain JCM. Fractions eluting from the chromatograph were collected at 0.5 min intervals and measured for radioactivity as described in Methods. **B** Elution profile of biphenyl metabolites produced by *Oscillatoria* sp., strain JCM. Fractions eluting between 17—20 min were pooled and used in the experiment described in Fig. 3. **C** Elution profile of synthetic hydroxybiphenyls. All separations were achieved using a 3.9 mm × 30 cm μBondapak C_{18} column with a linear gradient of 30—70% acetonitrile in water over 30 min. The initial flow rate was 1.5 ml/min

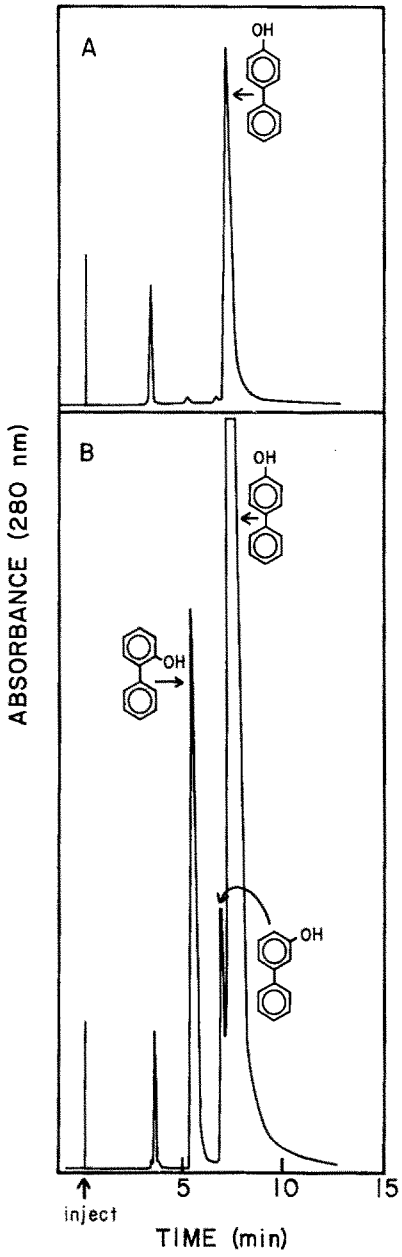

Fig. 3A and B. Hplc separation of monohydroxylated biphenyl isomers. **A** Metabolites produced by *Oscillatoria* sp., strain JCM. **B** Separation of synthetic 2-, 3-, and 4-hydroxybiphenyl. Separation was achieved with a 3.9 mm × 30 cm μPorasil column using hexane/ethyl acetate (85 : 15 v/v) as the eluting solvent (1.0 ml/min). Compounds eluting from the column were detected by *absorbance* at 280 nm

Results

When *Oscillatoria* sp., strain JCM was grown photoautotrophically in the presence of [^{14}C]-biphenyl, autoradiographic tlc analysis indicated that at least 3 metabolites were formed (Fig. 1). The major radiolabelled metabolite (Compound I) co-chromatographed with authentic 4-hydroxybiphenyl. The minor meta-

bolites (Compounds II and III) co-chromatographed with 2-hydroxybiphenyl and 4,4,-dihydroxybiphenyl respectively. Similar experiments with substrate controls showed no oxidation products. When the experiment was repeated and the ethyl acetate extract analyzed by hplc similar results were obtained (Fig. 2A). The major portion of radioactivity in the metabolic region of the chromatographic profile co-eluted with 4-hydroxybiphenyl. A small amount of radioactivity was also found which co-chromatographed with 4,4′-dihydroxybiphenyl. For comparative purposes the chromatographic mobilities of several known biphenyl metabolites are also shown in Fig. 2C. The amount of biphenyl metabolized to organic soluble metabolites was 2.9% of the total added [^{14}C]-biphenyl.

In order to obtain sufficient quantities of biphenyl metabolites for identification purposes five flasks were incubated with biphenyl as described in methods and the organic extracts were pooled. Hplc analysis of the pooled extracts revealed the presence of a major product with an identical retention time (18.5 min) to that given by 4-hydroxybiphenyl (Fig. 2B). Although the hplc data indicates that 4-hydroxybiphenyl is the predominant metabolite, the reverse-phase chromatographic system described does not separate 4-, and 3-hydroxybiphenyl. A normal phase hplc system was used to achieve this separation (Fig. 3B). Fractions that eluted with the same retention time as 4-hydroxybiphenyl (Fig. 2B) were pooled, conentrated and analyzed using the normal phase chromatographic system (Fig. 3A). The major metabolite had identical retention time (7.5 min) to synthetic 4-hydroxybiphenyl. In addition the absorption spectrum (λmax methanol, 260 nm) and mass spectrum (parent ion, *m/e* 170) of the metabolite was identical to authentic 4-hydroxybiphenyl. As can be seen in Fig. 3A a minor compound was detected that had an identical retention time (7.0 min) to authentic 3-hydroxybiphenyl but insufficient material was available for further structural identification.

Discussion

The results indicate that a cyanobacterium, *Oscillatoria* sp., strain JCM oxidizes biphenyl predominantly at the 4-position to form 4-hydroxybiphenyl. Although other metabolites were detected by autoradiography and hplc, they were not formed in sufficient amounts for structural identification. The site of hydroxylation is of interest since it is well documented that bacteria oxidize biphenyl at the 2,3-position to form cis-2,3-dihydro-2,3-dihydroxybiphenyl which is then oxidized to 2,3-dihydroxybiphenyl. That latter compound is the substrate for fission of the aromatic nucleus (Lunt and Evans, 1970; Catelani et al., 1971, 1973; Catelani and Colombi, 1974; Ohmori et al., 1973; Gibson et al., 1973). The formation of 4-hydroxybiphenyl as the major metabolite by *Oscillatoria* sp., strain JCM is similar to results observed in fungal (Smith and Rosazza, 1974; Wiseman et al., 1975; Dodge et al., 1979) and mammalian (Raig and Ammon, 1970; Meyer and Scheline, 1976; Wiebkin et al., 1976; Meyer et al., 1976) biotransformation studies.

4-Hydroxybiphenyl could be formed by an arene oxide mechanism. For example, a monooxygenase catalyzed reaction could form biphenyl 3,4-expoxide which then would isomerize to 4-hydroxybiphenyl. This pathway has been suggested for mammalian systems (Billings and McMahon, 1978). The formation of 4-hydroxybiphenyl could also be explained by a light-dependent direct oxygen insertion mechanism.

Research is now in progress to elucidate the mechanism of hydroxylation and the enzymes involved in these reactions. The results reported in this paper are an extension of our study on the metabolism of aromatic hydrocarbons by cyanobacteria and microalgae. Previously we showed that *Oscillatoria* sp., strain JCM oxidized naphthalene predominantly to 1-naphthol (Cerniglia et al., 1980a). Also *cis*-1,2-dihydroxy-1,2-dihydronaphthalene and 4-hydroxy-1-tetralone were formed. In this study *Oscillatoria* sp., strain JCM oxidized biphenyl to 4-hydroxybiphenyl. From these observations it is clear that cyanobacteria have the capacity to metabolize aromatic hydrocarbons. It will be interesting to elucidate the role of these organisms in the fate of aromatic hydrocarbons in the marine environment.

Acknowledgements. This investigation was supported by Grant Number ES-00537 awarded by The Institute of Environmental Health Sciences, DHEW. CEC is a postdoctoral trainee supported by Grant Number T32 CA09182 awarded by the National Cancer Institute, DHEW. We thank Rita O'Donnell for technical assistance and Eva Martin and Roberta DeAngelis for assistance in preparing the manuscript.

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Received October 15, 1979